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Synthesis of the First Ferrocene-Labeled Dideoxynucleotide and Its Use for 3′-Redox End-Labeling of 5′-Modified Single-Stranded Oligonucleotides

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The target ferrocene-labeled dideoxynucleotide compound 5-[*N*-(β-ferrocenyl-propanoyl)3-amino-propyn-1-yl]-2′,3′-dideoxyuridine 5′-triphosphate, Fc-ddUTP, was synthesized and tested with terminal deoxynucleotidyl transferase for enzymatic 3′-redox-active end-labeling of 5′-phosphorylated single-stranded oligodeoxynucleotides. Starting from readily available 5-iodouridine and 3-ferrocenylpropanoic acid, the synthetic strategy elaborated here follows a mild multistep route. Each step involves reliable methods, and all ferrocene intermediates can be easily purified. Enzymatic 3′-ferrocene end-labeling of 5′-phosphorylated oligonucleotides is remarkably efficient, and 3′-ferrocene-labeled oligonucleotides can thus be prepared in sufficient amounts for further use in surface modifications.

Redox-active labeling of DNA has received recent substantial attention (*1*–*14*). In this regard, ferrocene-ended single stranded DNA oligomers have emerged as important versatile tools for the investigation of nucleic acids. In particular, ferrocene–oligodeoxynucleotide (ODN) conjugates have been recognized as viable alternatives to the use of radioactive isotopes in traditional DNA sequencing techniques, and their usefulness as hybridization redox probes for electrochemical sensing of target DNA/RNA sequences has been demonstrated in a number of modern applications (*6*–*11*). More specifically, ferrocene-labeled ODNs are routinely finding utility in the development and miniaturization of bioelectronic gene-sensing systems (*12, 13*).

A great, yet nonexploited, potential lies in the development of ferrocene-ended DNA probes bearing at the other terminus a function able to create a stable attachment to solid supports, surfaces and biomolecules. To our knowledge, only one group has been interested to address this promising research area. In this context, Letsinger et al. can be acknowledged for the pioneering synthesis of a 5′-ferrocene-nucleotide 3′-thiol-ended adsorbate for chemisorption onto gold surfaces and characterization of a redox-active nucleotide monolayer (*14*). However, despite the potential interest recognized by the authors in preparing redox-active oligonucleotide molecular assemblies on well-defined surfaces, it appears that the proposed chemical synthetic strategy for introducing a ferrocene label and a chemical functionality within an individual nucleotide was not expanded any further to oligonucleotides. Their strategy which focuses primarily on the construction of a ferrocene-labeled phosphoramidite for subsequent coupling to a nucleotide by standard automated DNA solid-phase synthesis suffers from a serious drawback: the ferrocene subunit does not suitably withstand the chemical oxidation step conditions required (iodine in basic THF/H_2O) in the coupling cycle. As noted in ref 14, the bound-ferrocene is readily converted into its corresponding ferrocenium form. However, in the absence of an appropriate reductant, ferrocenium cations are well-known to rapidly hydrolyze in neutral/basic media with only partial regeneration of the parent ferrocene (*15*–*17*). Therefore, failure sequences lacking the ferrocene function inevitably are formed as byproducts, thus affecting product yield and reproducibility. While the phosphoramidite method generally offers the versatility of incorporating a metal complexe at any site along the synthetic ODN (*3, 18*), postmodifications of a functionalized oligonucleotide after standard solid-phase synthesis are more appropriate approaches for incorporation of the sensitive ferrocene functionality (*6, 10*).

As part of an ongoing program on the investigation of the dynamic properties of biopolymer chains end-tethered to surfaces (*19, 20*), our interest was focused on developing redox-active ferrocene end-labeled DNA oligomers having a transformable monophosphate group at the 5′-end. The phosphate at this terminus is of importance since it can be used directly for ready attachment to amino-bearing surfaces through phosphoramidate linkages while preserving the integrity of the DNA single-strand sequence. Moreover, this 5′-end group can be profitably exploited for diverse bioconjugations and extension modifications (*21, 22*). Our approach to these complex labeled oligonucleotides combines key features that make it well-suited for preparation of 3′-ferrocene-5′-phosphorylated oligonucleotides that vary considerably in nature and length, from short ODNs accessible by chemical synthesis to enzymatically synthesized long nucleic acid sequences. Specifically, the route involves enzymatic 3′-end-labeling of presynthesized 5′-phosphorylated oligonucleotides that leaves the 5′-end intact and makes use of terminal deoxynucleotidyl transferase (terminal transferase, TDT). Terminal transferase catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3′-OH ends of nucleic acids (*23, 24*). The enzyme accepts also a number of deoxynucleoside triphosphates containing modified base and/or sugar moieties. In this regard, C-5 labeled uridine 2′,3′-dideoxynucleotides (ddUTPs) comprise a unique class of monomer substrates that are compatible with polymerase enzymes, and due to lack of the 3′-hydroxyl

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functionality on the 2′-deoxyribose, offer a unique way to add a single label group such as biotin and digoxigenin (*25, 26*) or fluorophore groups (*27, 28*), to a DNA fragment. These labeled nucleotides are to date critical compounds in DNA sequencing techniques and antiviral chemotherapy (*29, 30*), as well as in molecular biology and biochemistry studies. Thus it seemed to us quite reasonable to expect a tolerance of terminal transferase to modification of the uracyl base moiety with a ferrocene unit.

As noted recently in a comprehensive review, no general and high-yielding route for preparing 2′,3′-dideoxynucleosides 5′-triphosphates is currently available, but a renewed interest in the synthesis of these important compounds is highly expected (*31*). We felt that application of modern synthetic organic methodologies utilized in 2,3′-dideoxyuridines (ddUs) chemistry (*29, 30*) coupled with recent methods of analysis for the detection and isolation of nucleoside triphosphates would lead to a useful synthetic approach to ferrocene-labeled 2′,3′-dideoxynucleoside triphosphates. In this paper, we describe the complete chemical synthesis of a first representative of the series, 5-[*N*-(β-ferrocenyl-propanoyl) 3-amino-propyn-1-yl]-2′,3′-dideoxyuridine 5′-triphosphate, Fc-ddUTP, from inexpensive 5-iodo-2′-deoxyuridine. All reactions utilize readily available reagents and standard laboratory conditions. We further demonstrate that Fc-ddUTP is an excellent substrate for terminal deoxynucleotidyl transferase, a commercially available enzyme, and can be employed for a routine preparation of redox active 3′-end-labeled DNA probes.

EXPERIMENTAL SECTION

Ferrocenylmethyltrimethylammonium iodide was purchased from Strem Chemicals and from Aldrich Chemical Co. 5-Iodo-2′-deoxyuridine and propargylamine were purchased from Aldrich Chemical Co. Tetra-*n*-butylammonium dihydrogenophosphate ($NBu_4KH_2PO_4$) (99+%) was purchased from Fluka. Tri-*n*-butylammonium pyrophosphate ($(HNBu_3)_2H_2P_2O_7$) was obtained from Sigma Chemical Co. Other chemicals were obtained from commercial sources and were reagent grade or better quality and used as received. All solvents were anhydrous unless otherwise noted.

Terminal deoxynucleotidyl transferase (EC 2.7.7.31), TDT, and tailing reaction buffer were purchased from Roche Molecular Biochemicals (Boehringer, Mannheim). Synthetic 5′-phosphorylated oligonucleotides (sodium salts) were purchased from ESGS Cybergene (Evry, France) and were HPLC purified grade products. Centricon-30 YM cellulose membranes were purchased from Amicon.

Unless otherwise specified, all reactions were carried out in oven-dried glassware under argon atmosphere. All the ferrocene compounds prepared in this work were stored in vacuo (over P_2O_5) at ambient temperature, protected from light, and dried for at least 12 h before first use.

Chromatography. RP high-performance liquid chromatography (HPLC) was performed on a system equipped with two Gilson HPLC pumps (Model 305 and 306), a Gilson 119 UV−vis detector operating at 260 or 270 nm. Pump control and data processing used a Rainin Dynamax chromatographic software package (Macintosh).

For nonionic ferrocenes, analytical HPLC was carried out on a Waters (Milford, MA) Novapak C_{18} (4 μm, 3.9 mm i.d. × 15 cm) column with a linear gradient elution from 35% to 75% acetonitrile in water in 30 min (system I). For other analytical runs, HPLC solutions were as follows: solvent A, 10% acetonitrile in aqueous KH_2PO_4 (20 mM) and $NBu_4KH_2PO_4$ (5 mM), pH adjusted to 7.5 with KOH; solvent B, acetonitrile. HPLC on a Novapak C_{18} was performed isocratically with 25% B (system II) or following a linear gradient elution from 10 to 40% B in 60 min (system III). Purification of ferrocene-end-labeled oligonucleotides was carried out by HPLC on a Nucleosil 120C_{18} (5 μm, 4.6 mm i.d. × 15 cm) column (20 μL loop) following a linear gradient elution from 25% to 35% B in 20 min (system IV). For other experiments performed on a Nucleosil 120C_{18}, the following conditions were used: solvent A, 10% acetonitrile in aqueous triethylammonium acetate, Et_3NH^+, AcO^- (TEAA, 0.1 M) pH adjusted to 7.0 with acetic acid; solvent B, acetonitrile, (100 μL loop). Elutions were done with a linear gradient from 2.5% to 25% B over 30 min (system V). For all HPLC runs the flow rate was 0.8 mL/min.

Thin-layer chromatography (TLC) analyses were performed on 0.25 mm silica gel Polygram SIL G/UV_{254} plates. Preparative column chromatography was carried over Magerey-Nagel silica gel (0.063−0.2 mm), or over Sigma Chemical Co. ion-exchange DEAE cellulose or DOWEX 50WX4−100 (H^+ form, strongly acidic) resins.

A 1 M solution of triethylammonium hydrogenocarbonate Et_3NH^+,HCO_3^- (TEAC) was prepared by bubbling CO_2 through an aqueous mixture of triethylamine until the organic layer disappeared and adjusted to pH 7.5 with Et_3N.

Spectrometries. ^1H and proton-decoupled ^{13}C NMR spectra were recorded on a Bruker AM-200 spectrometer operating at 200 and 50.3 MHz, respectively, in $CDCl_3$, CD_3OD, and /or DMSO-d_6. Chemical shifts are reported in parts per million relative to the solvents' residual protons $CDCl_3$ δ 7.27 ppm, CD_3OD, 3.31 ppm, DMSO-d_6 2.50 ppm for ^1H experiments and the center line of $CDCl_3$ (77.00 ppm) CD_3OD (49.00) for ^{13}C NMR experiments. ^{31}P NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 161.9 MHz in D_2O and referenced to 85% H_3PO_4.

Fast atom bombardment (FAB) mass spectra were obtained in the negative ion mode from the Ecole Normale Supérieure, Département de Chimie (Paris, France) using "magic bullet" (MB) as matrix. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a PerSeptive Biosystems Inc. Voyager Elite instrument, operating in the negative mode using 3-hydroxypicolinic acid in diammonium citrate as the matrix and 355-nm pulsed light (*49*). External calibration was carried out using HPLC desalted 5′-p-$(dT)_{10}$ (system V) in the above matrix.

UV−visible spectra and optical density (OD) were measured on a Hewlett-Packard HP 8452 diode array spectrophotometer. The quantity and yield of the ferrocene-nucleotides was determined by measuring the OD_{290} of aqueous solutions of ferrocene-nucleotides and taking the value of ϵ_{290} for Fc-ddU **7** (ϵ_{290} = 9500 M^{-1} cm^{-1}) as a standard value for **8**−**11**.

3-Ferrocenylpropanoic Acid (1). 3-Ferrocenylpropanoic acid **1** was prepared from ferrocenylmethyltrimethylammonium iodide following an adaptation of the three-step procedure reported in the past (*50*). The main modification is that ferrocenylmethylmalonic diethyl ester obtained in the first step was rigorously purified by fractional distillation before use. Without that additional purification step, we have found that the ferrocene monocarboxylic **1** could not be obtained as a clean sample.

A solution of diethyl malonate (8.8 g, 55 mmol) in 25

mL of absolute ethanol was added dropwise to a well-stirred solution containing freshly cut sodium (1.26 g, 55 mmol) in 50 mL of absolute ethanol. After complete addition, ferrocenylmethyltrimethylammonium iodide (19.3 g, 50 mmol) was added. The solution was heated under reflux for 72 h. After cooling to ambient temperature, the reaction mixture was diluted with water (200 mL), neutralized with 1 N HCl, and then extracted with two 100-mL portions of diethyl ether. The combined extracts were washed with saturated aqueous sodium hydrogenocarbonate, dried over magnesium sulfate, filtered, and evaporated to give an oily residue that was subjected to fractional distillation under reduced pressure. Discarding the first colorless fraction corresponding to residual diethyl malonate, the desired ferrocenylmethylmalonic diethyl ester was distilled as an orange oil (3 mmHg, 139−141 °C) which slowly solidified on standing (12.1 g, 68%). ^{1}H NMR ($CDCl_3$) δ 4.19 (q, 4H, OCH_2), 4.13 (s, 5 H, CpH), 4.08 (m, 4 H, CpH), 3.46 (t, J = 7.45 Hz, 1H, CH), 2.97 (d, J = 7.45 Hz, 2H, FcCH_2), 1.25 (t, J = 7.13 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$) δ 168.87, 84.55, 68.52, 67.57, 61.24, 54.15, 29.15, 13.96.

Ferrocenylmethylmalonic ester (10.7 g, 30 mmol) was saponified in 25 mL of 6 N potassium hydroxyde in 90% aqueous ethanol under reflux for 24 h. The reaction mixture was diluted with water (150 mL) and extracted with two 100-mL portions of diethyl ether. The aqueous phase was neutralized with 6 N HCl. After addition of diethyl ether (150 mL), the mixture was cautiously acidified with 6 N HCl under stirring until extraction with ether. The organic extract was washed with water, dried over magnesium sulfate, and evaporated to obtain 2-ferrocenylmethyl malonic acid (8.2 g, 90%). ^{1}H NMR (DMSO-d_6) δ 12.73 (br s, 2H, COOH), 4.2−4.0 (m, 9H, CpH), 3.32 (t, J = 7.33 Hz, 1H, CH), 2.79 (d, J = 7.33 Hz, 2H, FcCH_2).

A sample of 2-ferrocenylmethyl malonic acid (7.6 g, 35 mmol) was heated at 150−160 °C for 0.5 h. The resulting residue was then purified by column chromatography on silica gel in chloroform/methanol, $CHCl_3$/MeOH (95:5) to afford analytically pure 3-ferrocenylpropanoic acid **1** (6.0 g, 93%). mp 121−122 °C (116−118 °C (*50*), 119−120 °C (*51*)). ^{1}H NMR ($CDCl_3$) δ 4.2−4.05 (m, 9H, CpH), 2.75−2.55 (m, 4H, FcCH_2CH_2). ^{13}C NMR (DMSO-d_6) δ 174.08, 87.73, 68.30, 67.67, 66.94, 34.98, 24.31.

5-*N*-(β-Ferrocenyl-propanoyl)-3-amino-1-propyne (2). To a stirred solution of ferrocene **1** (5.16 g, 20 mmol) in 200 mL of dichloromethane were added 1-hydroxybenzotriazole (HOBT) (270 mg, 2 mmol) and propargylamine (2.06 mL, 40 mmol). After addition of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochoride, EDC (4.22 g, 22 mmol), the mixture was allowed to react under stirring for 24 h at ambient temperature. The solution was successively washed with three 100-mL portions of 0.5 M H_2SO_4, water (100 mL), and saturated aqueous sodium hydrogenocarbonate. The organic phase was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The solid residue was chromatographed on silica gel in $CHCl_3$/MeOH (95:5), yielding analytically pure ferrocene conjugate **2** (5.54 g, 94%). mp 78−79 °C. ^{1}H NMR ($CHCl_3$) δ 5.56 (br s, 1H, CONH), 4.3−3.9 (m, 11H, CpH + NHCH_2),), 2.70 (m, 2H, FcCH_2), 2.38 (m, 2H, CH_2CO), 2.23 (t, J = 2.55 Hz, 1H, CH). ^{13}C NMR (CD_3OD) δ 175.14, 88.79, 80.60, 72.12, 69.48, 68.96, 68.30, 38.27, 29.39, 26.63.

5-[*N*-(β-Ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2′-deoxyuridine (3) (Fc-dU). To a stirred solution of 5-iodo-2′-deoxyuridine (3.54 g, 10 mmol) in 50 mL of degassed DMF were added cuprous iodide (CuI) (38 mg, 0.2 mmol) and ferrocene **2** in excess (7.38 g, 25 mmol). The solution was stirred for 30 min at ambient temperature at which time triethylamine (Et_3N) (2.78 mL, 20 mmol) and tetrakis(triphenylphosphine)palladium(0) $(PhP_3)_4Pd$ (116 mg, 0.1 mmol) were added. After stirring of the solution for 48 h, CuI (0.2 mmol) and $(PhP_3)_4Pd$ (0.1 mmol) were added anew followed by addition of Et_3N (20 mmol). The reaction mixture was allowed to stir for another 24 h. At this point, TLC ($CHCl_3$/MeOH (85:15)) indicated quantitative conversion of the nucleoside reagent (R_f 0.42) to give the desired nucleoside−ferrocene product **3** (R_f 0.51). The solution was concentrated under vacuum at a temperature maintained at ≤35 °C, and the liquid residue was separated on silica gel. A first yellow fraction containing remaining starting ferrocene was eluted with $CHCl_3$/MeOH (95:5). A second yellow fraction was eluted with $CHCl_3$/MeOH (85:15), affording the desired product contaminated with a small amount of triethylammmonium iodide. Cristallization from acetonitrile yielded analytically pure **3** (3.76 g, 72%). mp 196−198 °C (with some decomposition). ^{1}H NMR (CD_3OD) δ 8.30 (s, 1H, H-6), 6.24 (app t, J = 6.6 Hz, 1H, H-1′), 4.40 (m, 1H, H-3′), 4.2−4.0 (m, 11H, CpH + NHCH_2), 3.94 (m, 1H, H-4′), 3.85−3.70 (m, 2H, H-5′), 2.65 (m, 2H, FcCH_2), 2.42 (m, 2H, CH_2CO), 2.4−2.1 (m, 2H, H-2′). ^{13}C NMR (CD_3OD) δ 175.16, 164.52, 151.11, 145.38, 99.94, 90.07, 89.15, 88.80, 87.03, 75.18, 72.07, 69.50, 68.97, 68.34, 62.62, 41.71, 38.36, 30.43, 26.63. HPLC (system I), retention time, t_r = 5.88 min.

A second chromatography using chloroform was performed on crude collected ferrocene **2** and allowed recovery of 2.95 g (67% from starting excess) of an essentially pure sample which could be used without further purification for a next preparation of **3**.

5′-O-(*tert*-Butyldimethylsilyl)-5-[*N*-(β-ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2′-deoxyuridine (4) (Fc-dU-5′TBDMS). Ferrocene−nucleoside **3** (1.56 g, 3 mmol) and imidazole (0.51 g, 7.5 mmol) were dissolved in 30 mL of DMF under stirring. To the solution was added *tert*-butyldimethylsilyl chloride (0.57 g, 3.75 mmol). The reaction mixture was stirred with the exclusion of moisture at ambient temperature for 1 h. The solvent was removed under vacuum at a temperature maintained at ≤ 35 °C, and the yellow residue was purified by column chromatography on silica gel in ethyl acetate/cyclohexane (95:5). Isolation of the major yellow component afforded the desired 5′-monosilylated ferrocene-nucleoside **4** as an orange solid (1.62 g, 85%). mp 165−166 °C. ^{1}H NMR (CD_3OD) δ 8.12 (s, 1H, H-6), 6.24 (dd, J = 7.45, 6.00 Hz, 1H, H-1′), 4.46 (m, 1H, H-3′), 4.2−4.0 (m, 12H, CpH + NHCH_2 + H-4′), 3.94 (dd, J = 11.50, 2.33 Hz, 1 H, H-5′), 3.84 (dd, J = 11. 50, 2.59 Hz, 1H, H-5′), 2.66 (m, 2H, FcCH_2), 2.40 (m, 2H, CH_2CO), 2.4−2.1 (m, 2H, H-2′), 0.97 (s, 9H, *t*-BuSi), 0.19 (m, 6H, CH_3Si). ^{13}C NMR (10% DMSO-d_6 in CD_3OD) 174.28, 163.85, 150.83, 144.35, 99.89, 90.67, 89.22, 89.02, 87.00, 75.60, 72.54, 69.56, 68.95, 68.33, 64.57, 42.22, 38.11, 30.27, 26.74, 26.44, 16.33, −4.90, −5.02. HPLC (system I) t_r = 19.49 min.

5′-*O*-(*tert*-Butyldimethylsilyl)-3′-O-(phenoxythiocarbonyl)-5-[*N*-(β-ferrocenyl-propanoyl) 3-amino-propyn-1-yl]-2′-deoxyuridine (5). (Fc-dU-5′TBDMS-3′PTC). To a solution of **4** (1.50 g, 2.36 mmol) in 3 mL of dichloromethane containing 1.4 mL of pyridine (17.3 mmol), cooled to 0 °C, was added dropwise a solution of phenoxythiocarbonyle chloride (0.94 mL, 6.84 mmol) in 3 mL of dichloromethane over a period of 30 min. After complete addition, the reaction mixture was stirred at ambient temperature for 2 h. At this point, TLC (ethyl acetate/cyclohexane (80:20)) showed the complete absence

of the starting nucleoside **4** (R_f 0.27) and its replacement by the thioacylated nucleoside **5** (R_f 0.63) and a byproduct (R_f 0.83). The solution was diluted with 100 mL of dichloromethane, washed successively with three 100-mL portions of 0.5 M H_2SO_4, water, and brine. After drying over magnesium sulfate, the solvent was removed under vacuum to give a solid residue which was chromatographed on silica gel. Elution with ethyl acetate/cyclohexane (95:5) afforded the desired product **5** (1.10 g, 60%) as an orange amorphous solid. ^{1}H NMR (CD_3OD) δ 8.13 (s, 1H, H-6), 7.5–7.0 (m, 5H, ArH), 6.32 (dd, J = 8.7, 5.5 Hz, 1H, H-1′), 5.79 (app d, J = 5.6 Hz, 1H, H-3′), 4.46 (m, 1H, H-4′), 4.15–4.0 (m, 13H, CpH + NHCH_2 + H-5′), 2.65 (m, 2H, FcCH_2), 2.41 (m, 2H, CH_2-CO), 2.9–2.2 (m, 2H, H-2′), 0.97 (s, 9H, t-BuSi), 0.19 (s, 3H, CH_3Si), 0.17 (s, 3H, CH_3Si). HPLC (system I) t_r = 34.22 min.

5′-*O*-(*tert*-Butyldimethylsilyl)-5-[*N*-(β-ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2′,3′-dideoxyuridine (6) (Fc-5′TBDMS-ddU). A well-stirred mixture of **5** (1.10 g, 1.42 mmol) and tributyltin hydride (0.42 mL, 1.56 mmol) in 70 mL of benzene was degassed for 30 min. After addition of 2,2′-azobis(2-methylpropionitrile) (AIBN) (23 mg, 0.14 mmol), the stirring reaction mixture was heated to reflux for 90 min. Solvent removal under reduced pressure gave a residue which was chromatographed on silica gel in ethyl acetate/cyclohexane (80:20), yielding the expected deoxygenated product **6** (0.46 g, 52%) as an orange amorphous solid. ^{1}H NMR (CD_3OD) δ 8.19 (s, 1H, H-6), 5.99 (m, 1H, H-1′), 4.2–4.0 (m, 13H, CpH + NHCH_2 + H-4′ + H-5′), 3.77 (dd, J = 11.7, 2.81 Hz, 1H, H-5′), 2.65 (m, 2H, FcCH_2), 2.41 (m, 3H, CH_2-CO + H-2′), 2.05–1.95 (m, 3H, H-2′ + H-3′), 0.97 (s, 9H, t-BuSi), 0.17 (s, 6H, CH_3Si). HPLC (system I) t_r = 25.89 min.

5-[*N*-(β-Ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2′,3′dideoxyuridine (7) (Fc-ddU). Product **6** (0.80 g, 1.29 mmol) was reacted with tetrabutylammonium fluoride in THF (1 M solution, 6.4 mL, 6.4 mmol) for 30 min. At this point, TLC (ethyl acetate–cyclohexane (80:20)) indicated complete disappearance of the starting nucleoside **6** (R_f 0.45) into the desired deprotected product **7** (R_f 0.07). After solvent removal, the residue was chromatographed on silica gel in $CHCl_3$/MeOH (90:10). Fractions containing **7** were combined and concentrated under vacuum to give a viscous liquid which was taken up in 100 mL of acetonitrile. After washing with six 100 mL portions of cyclohexane which removed silylated byproducts, the acetonitrile phase was evaporated under vacuum yielding **7** (0.60 g, 92%) as an amorphous orange solid. ^{1}H NMR (CD_3OD) δ 8.44 (s, 1H, H-6), 6.01 (dd, J = 6.6, 3.0 Hz, 1H, H-1′), 4.2–4.0 (m, 12H, CpH + NHCH_2 + H-4′), 3.91 (dd, J = 12.2, 2.9 Hz, 1H, H-5′), 3.69 (dd, J = 12.2, 3.5 Hz, 1H, H-5′), 2.65 (m, 2H, FcCH_2), 2.41 (m, 3H, CH_2CO + H-2′), 2.2–1.9 (m, 3H, H-2′ + H-3′). ^{13}C NMR (CD_3OD) δ 175.03, 164.65, 151.09, 145.56, 99.11, 89.81, 88.81, 88.20, 83.32, 75.43, 69.50, 68.94, 68.32, 63.26, 38.30, 33.85, 30.50, 26.56, 25.41. HPLC (system I) t_r = 7.81 min.

5-[*N*-(β-Ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2′,3′-dideoxyuridine 5′-monophosphate (9) (Fc-ddUP) as Morpholinium Salt. A 0.4 M stock solution of β-cyanoethyl phosphate pyridinium salt in pyridine was prepared by the procedure of Tener (*46*).

Nucleoside-ferrocene **7** (300 mg, 0.6 mmol) was dissolved in 3 mL of degassed anhydrous pyridine containing β-cyanoethyl phosphate in excess (3 mL of the stock solution, 1.2 mmol). To this solution was added dicyclohexylcarbodiimide (DCC) (495 mg, 2.4 mmol). The reaction mixture was stirred at ambient temperature for 48 h. At this point, HPLC (system II) showed the quantitative conversion of the starting nucleoside (t_r = 7.1 min) into the less hydrophobic β-cyanoethyl phosphate derivative **8** (t_r = 6.5 min). To the solution was added 12 mL of water, and the mixture was stirred at ambient temperature for 1 h. The precipitate of *N,N*-dicyclohexylurea was removed by filtration and washed well with water. The filtrate and washings were combined and concentrated under vacuum. The resulting residue was purified from excess undecomposed phosphorylating reagent by chromatography on DEAE cellulose using 0.1 M triethylammonium hydrogenocarbonate (TEAC) as the eluant. The yellow eluate was collected and lyophilized to afford intermediate **8** as a chromatographically pure solid: ^{31}P (D_2O) δ 0.96 (s, 1P, P_α)

The solid was dissolved in 30 mL of 9 N aqueous ammonia, and the solution was heated at 55 °C for 1 h. At this point, HPLC (system II) indicated conversion of the intermediate **8** into the expected monophosphate product **9** Fc-ddUP (t_r = 4.6 min) contaminated as shown by ^{31}P NMR spectroscopy with what appeared to be the corresponding diphosphate derivative. The solution was concentrated under vacuum and the residue was passed onto a DEAE (HCO_3^- form). The column was eluted with a linear gradient of TEAC (0.0 to 0.4 M, pH 7.5). Appropriate fractions were dried by lyophilization, and excess salt was removed by repeated lyophilization with deionized water to yield the triethylammonium salt of Fc-ddUP. An aqueous solution of Fc-ddUP was then applied to a Dowex 50 WX4 cation-exchange column (morpholinium form) and eluted with H_2O. The solution was lyophilized to afford morpholinium ferrocene–2′,3′-dideoxyuridine phosphate **9.** The isolated yield (58%, based on starting **7**) was determined by optical density measurement. Attempts to separate the product from a phosphorus impurity gave the monophosphate compound, still not completely pure. The content of "morpholine" was determined to be 1.1 equiv by ^{1}H NMR. ^{1}H NMR (CD_3OD) δ 8.32 (s, 1H, H-6), 6.01 (m, 1H, H-1′), 4.27 (m, 1H, H-4′), 4.20–4.05 (m, 11H, CpH + NHCH_2), 4.03 (m, 2H, H-5′), 3.79 (br s, 1.1 × 8H, CH_2 morpholinium), 2.65 (m, 2H, FcCH_2), 2.55–2.35 (m, 3H, CH_2CO + H-2′), 2.25–1.95 (m, 3H, H-2′ + H-3′). ^{13}C NMR (CD_3OD) δ 175.17, 164.71, 151.29, 145.39, 99.63, 90.08, 89.95, 88.10, 82.26 (d, J = 8.8 Hz, 1H, C-4′), 75.65, 69.51, 69.02, 68.29, 66.85 (d, $J_{C,P}$ = 4.8 Hz, 1H, C-5′), 65.98, 45.04 (morpholinium), 38.27, 33.18, 30.65, 26.62, 26.37. ^{31}P NMR (D_2O) δ 2.07 (s, 1P, P_α). HPLC (system II) t_r = 4.6 min. FAB-MS (–) data: *m/z* $(M - H)^-$, 584.0, $[C_{25}H_{27}N_3O_8PFe]H_3$ requires 584.1.

^{31}P NMR signals from what appears to be the diphosphate are as follows: δ −8.7 (d, 1P, J = 21 Hz, P_β), −13.2 (d, 1P, J = 21 Hz, P_α).

5-[*N*-(β-Ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2′,3′-dideoxyuridine 5′-triphosphate (9) (Fc-ddUTP) as Triethylammonium Salt. A solution of the monophosphate **9** (168 mg, 0.25 mmol) in water (2.5 mL) was mixed with *tert*-butyl alcohol (2.5 mL) and morpholine (85 μL, 1 mmol). To the stirred refluxing solution was added a solution of *N,N*-dicyclohexylcarbodiimide (206 mg, 1 mol) in *tert*-butyl alcohol dropwise over a period of 1.5 h. As shown by HPLC analysis, the quantitative formation of the phosphoromorpholidate **10** (system II, t_r = 6.2 min) was obtained after an additional 1 h. The reaction mixture was then cooled and evaporated under reduced pressure. The residue was dissolved in water (20 mL) and filtered; the solid was washed with an additional portion of water (5 mL). The combined

aqueous portions were extracted with diethyl ether (3 × 20 mL) and then coevaporated under reduced pressure with pyridine and finally benzene.

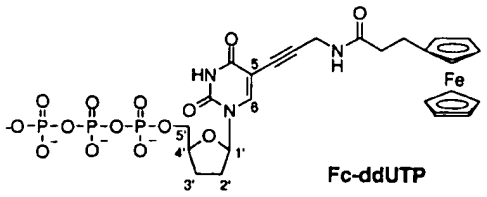
A solution of 47 mg of the above solid in dry DMSO (1.4 mL) was added slowly to a solution of tri-*n*-butylammonium pyrophoshate (101 mg, 0.28 mmol) in dry DMSO (0.4 mL), and the mixture was stirred under an inert atmosphere at ambient temperature for 3 days. The resulting solution was applied to a DEAE cellulose column (HCO_3^- form). The column was washed with water until the absorbance of the effluent fell to zero and then eluted with a linear gradient of triethylammonium hydrogenocarbonate (0.1 to 0.4 M). Appropriate fractions (0.2−0.3 M) monitored by HPLC for phosphorus content were pooled and lyophilized to yield Fc-ddUTP which was finally was purified by HPLC on Nucleosil C_{18} (system V). The isolated yield was determined by optical density measurement (0.12 mmol, 48%). The content of "triethylamine" was determined to be 3.2 equiv by ^{1}H NMR. ^{1}H NMR (CD_3OD) δ 8.24 (s, 1H, H-6), 6.01 (m, 1H, H-1′), 4.28 (m, 1H, H-4′), 4.20−4.05 (m, 11H, + CpH + NHCH_2), 4.04 (m, 2H, H-5′), 2.87 (m, 3.2 × 6 H, CH_2 triethylammonium), 2.63 and 2.48 (A_2B_2 system, 4H, FcCH_2CH_2-CO), 2.38 (m, 1H, + H-2′), 2.20−2.00 (m, 3H, H-2′ + H-3′), 1.18 (m, 3.2 × 3H, CH_3 triethylammonium). ^{31}P NMR (D_2O) δ −9.80 (d, 1P, J = 20 Hz, P_γ), −10.1 (d, 1P, J = 20 Hz, P_α),-22.1 (t, 1P, J = 20 Hz, P_β). HPLC (system II), t_r = 5.5 min. FAB-MS (−) data: *m/z* (M − H)$^-$, 744.0, $[C_{25}H_{27}N_3O_{14}P_3Fe]H_3$ requires 744.0; (M − 2H + Na)$^-$, 766.0, $[C_{25}H_{27}N_3O_{14}P_3Fe]H_2Na$ requires 766.0; (M − 3H + 2Na)$^-$, 788.0, $[C_{25}H_{27}N_3O_{14}P_3Fe]HNa_2$ requires 788.0.

5′-Phosphorylated Oligonucleotides Containing a 3′-Terminal ddU-Fc (5′-p-(dT)$_{10}$-ddU-Fc-3′). In a typical experiment, approximately 20 nmol of 5′-phosphorylated ODN 5′-p-(dT)$_{10}$ (23 μM) and 200 nmol of Fc-ddUTP (0.23 mM) in tailing buffer (850 μL) containing 25 mM Tris-HCl and 100 mM potassium cacodylate, pH 7.2, 1 mM $CoCl_2$ and 0.2 mM dithiothreitol were mixed with 500 units of terminal deoxynucleotidyl transferase (20 μL). The reaction was incubated for 30 min at 37 °C. The enzyme was then removed by filtration through a Centricon 30 YM cellulose membrane (30000 MW cutoff). For complete recovery of the ferrocene-labeled oligonucleotide, the membrane unit was washed by successive dilutions with water and filtrations (5 × 500 μL). The filtrates were concentrated by lyophilization and purified by HPLC using a linear gradient of sodium phosphate buffer (system IV) to remove reaction buffer components and excess Fc-ddUTP (t_r = 7.7 min). Appropriate fractions (t_r = 13.8 min) were collected in sterile Eppendorf tubes and were lyophilized to afford the oligonucleotide extended from the 3′-end with a ddU-Fc label (5′-p-(dT)$_{10}$-ddU-Fc-3′) in a ca. 75−80% yield. Quantitation was done from the HPLC peak integral at 270 nm, the molar extinction coefficient being the sum of the estimated molar extinction coefficient for 5′-p-(dT)$_{10}$ (ϵ_{270} = 97800 M^{-1} cm^{-1}) (*9*) and of the molar extinction coefficient of the Fc-ddU moiety (ϵ_{270} = 6640 M^{-1} cm^{-1}) which was determined separately. Finally, the purified ferrocene-labeled DNA probe was desalted using a linear gradient of TEAA in acetonitrile buffer (system V, t_r = 22.5 min) prior to MALDI-TOF analysis. MS (MALDI-TOF) (−) data: *m/z* (M − H)$^-$, 3623.8, $C_{125}H_{157}N_{23}O_{78}P_{11}Fe$ requires 3624.5.

RESULTS AND DISCUSSION

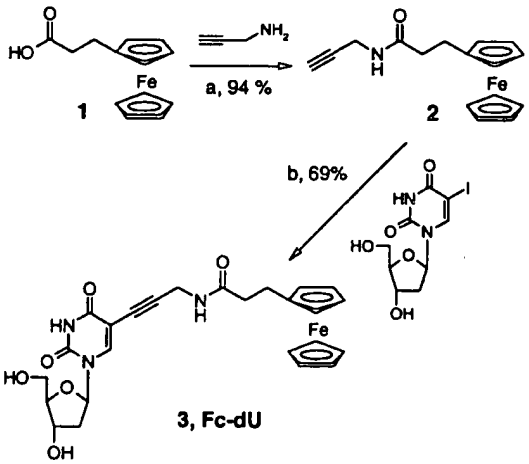
Synthesis and Characterization of C-5 Ferrocene Nucleosides and Nucleotides. Of major concern for successful 3′-end-labeling of single-stranded DNAs is the appropriate choice of the linkage between the label and the uridine base in the C-5 labeled nucleotide substrate. For the Fc-ddUTP species we chose to synthesize (Scheme 1), the precise positioning of the ferrocene label is ensured through a triple-bonded cross-linking to the base. This structural feature originally designed by Prober et al. (*32*) is actually found in a variety of commercially available C-5 modified chain terminators. While the rigid triple bond is expected to reduce the interaction between the label and the nucleotide, the alkynyl C=CH−CH_2−NH−C(O)−$(CH_2)_2$ arm we selected for Fc-ddUTP generally minimizes interference with enzyme activity. Choice of this linker arm would also offer an interesting redox-labeled nucleotide endowed with the low redox potential of an alkyl ferrocene derivative. Finally, because the C-5 positions of uridines are not involved in the Watson−Crick base pairing, the ferrocene label is expected not to interfere with probe hybridization, a criteria of major importance for development of valuable ferrocene-labeled DNA probes.

Scheme 1



Our synthetic approach to the target compound Fc-ddUTP follows a chemical route which consists of three main stages: (a) synthesis of a C-5 ferrocene-labeled 2′-deoxyuridine precursor Fc-dU (Scheme 2); (b) specific 3′-deoxygenation to Fc-ddU (Scheme 3); (c) triphosphorylation of the 5′-end via the monophosphate form Fc-ddUP (Scheme 4). This strategy capitalizes on well-established and mild methodologies in uridine chemistry that we anticipated to be compatible with the ferrocene unit. As a second main advantage, the route starts from inexpensive and readily available materials. In particular, 5-iodo-2′deoxyuridine was selected for modification because of its commercial availability and current use at the gram level. Additionally, incorporation of the ferrocene label facilitates the purification work because of its hydrophobicity and coloration, therefore providing a quick entry to a variety of new ferrocene-nucleosides.

Scheme 2[a]



[a] (a) EDC, HOBT, CH_2Cl_2; (b) $(PPh_3)_4Pd$, CuI, Et_3N, DMF.

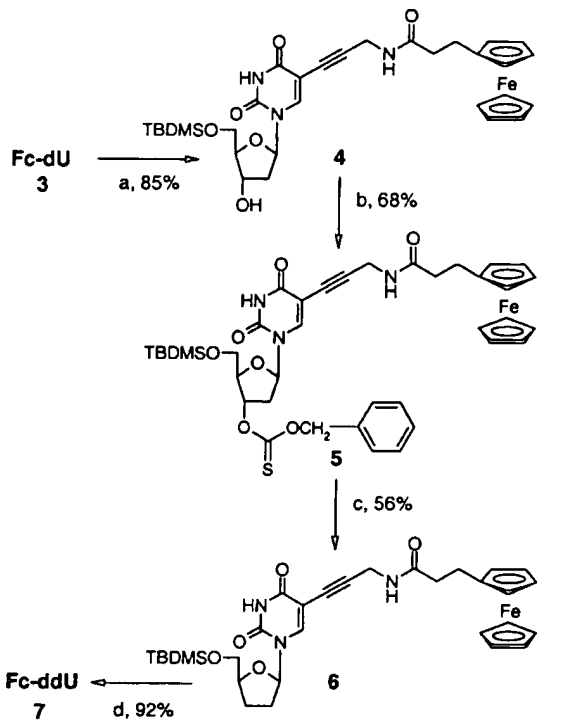
As outlined in Scheme 2, the synthetic stage to Fc-dU **3** relies on the classical palladium-mediated coupling methodology of terminal alkynes with 5-iodo-nucleosides. The starting 5-*N*-(β-ferrocenyl-propanoyl)-3-amino-1-propyne linker **2** was readily prepared by carbodiimide EDC-mediated condensation reaction of 3-ferrocenylpropanoic acid **1** with propargylamine in dichloromethane. The presence of 1-hydroxybenzotriazole (HOBT) as an additive was necessary for efficient reaction. This afforded **2** in an excellent isolated yield (94%) and purity. An improved synthesis of **1** is given in the Experimental Section.

Coupling of alkyne **2** with 5-iodo-2′-deoxyuridine was carried out in DMF following the Pd(0)-catalyzed methodology reported by Hobbs (*33*). It seemed from literature observations that formation of the cyclized furano pyrimidin-2-one byproduct which generally occurs under the Sonogashira Pd(II)-catalyzed coupling reactions (*33*–*35*) would be prevented. Indeed, at ambient temperature and using the $(PhP_3)_4Pd/CuI$ catalytic system, the coupling reaction proceeded cleanly to give FcdU as the sole ferrocene-containing nucleoside. Compound Fc-dU was readily isolated by silica gel chromatography followed by crystallization in 72% yield. Its structure and purity were confirmed by ^1H NMR and ^{13}C NMR spectra and reversed-phase (RP) HPLC.

Our route for specific chemical 3′-deoxygenation of ferrocene-2′-deoxyuridine Fc-dU to the corresponding ferrocene-2′,3′-dideouridine Fc-ddU involves the classical and mild four-step sequence: selective-protection of the 5′-hydroxyl function, thioacylation followed by radical-induced reductive cleavage, and deprotection (*36*) (Scheme 3). We chose to protect the 5′-hydroxy of Fc-dU with the *tert*-butyl-dimethyl silyl TBDMS group to avoid acid conditions during deprotection since 2′,3′ dideoxyuridines are acid labile (*37*, *38*). Thus, Fc-dU was treated with *tert*-butylchlorodimethylsilane (TBDMSCl) in the presence of imidazole in DMF to give the 5′-*O*-TBDMS derivative **4** which was obtained after chromatographic separation from minor amounts (<5%) of the 3′,5′-bis-silylated product in 85% yield. The removal of the 3′-hydroxy group of ferrocene-nucleoside **4** was basically achieved by adaptation of the Barton–McCombie deoxygenation procedure of alcohols (*39*) as modified by Robins et al. for nucleosides (*36*). Accordingly, **4** was converted in a first step into its 3′-(phenyl thionocarbonate) ester **5** using phenoxythiocarbonyle chloride (PTC-Cl).

However, it appeared that the acetonitrile/4-dimethylaminopyridine catalytic system classically used for thioacylation of hindered alcohols (*36*) was unsuitable for large-scale preparation of **5**. In addition to the limited solubility of **4** in acetonitrile, thioacylation of **4** was complicated by formation of a ferrocene–nucleoside byproduct in an approximate 50% amount. This product is believed to be the corresponding O^2,3′-anhydronucleoside derivative of **4** (Figure 1), judging from the marked downfield position of the H-3′ (δ 5.75 ppm) signal, slightly displaced 0.06 ppm upfield from the corresponding signal in the ester **5**, and the absence of the aromatic phenoxy signals in the NMR spectrum of a byproduct chromatography fraction. Similar O^2,3′-anhydronucleosides have been reported in the literature and can be obtained by direct treatment with various reagents of the appropriate 2′-deoxyuridine but also by base treatment of the corresponding 3′-*O*-thiocarbonyl-nucleoside precursor (*40*–*42*). The low yield and the difficulty of purification of the desired **5** led us to use alternate conditions for the 3′-thioacylation step of **4**. In dichloromethane and in the presence of ~7 mol equiv of pyridine (*43*), O^2,3′-cyclonucleoside formation could be reduced to less than 10%, possibly due to the shorter reaction times (<2 h). 3′-*O*-(Phenoxythiocarbonyl)ferrocene–nucleoside **5** was obtained as the major product and was isolated in this work in 62% (pure) as an amorphous solid.

Scheme 3[a]



[a] (a) *tert*-Butyldimethylsilyl chloride, DMF; (b) phenoxythiocarbonyl chloride, CH_2Cl_2, pyridine; (c) tributyltin hydride, AIBN, benzene; (d) TBAF, THF.

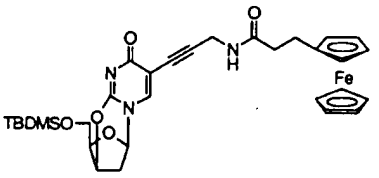
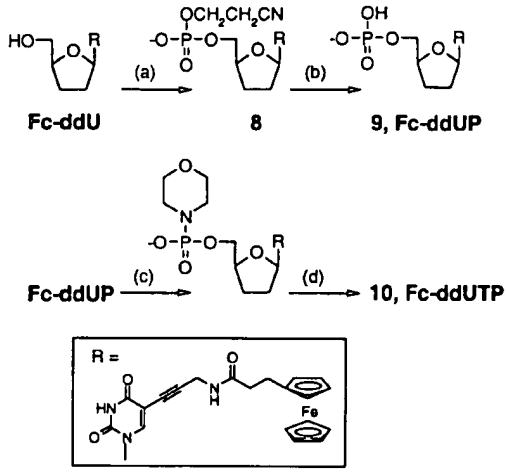


Figure 1. Ferrocene-cyclonucleoside byproduct formed during thioacylation of **4**.

Compound **5** was then smoothly deoxygenated with commercial tri-*n*-butyltin hydride (Bu_3SnH) and a catalytic amount of 2,2′-azobisisobutyronitrile (AIBN). The best chemical yield in the desired 2′,3′-dideoxyuridine product **6** was obtained after a short heating at benzene reflux under argon. Under such conditions, no dethiation or alcohol byproducts were detected. Interestingly, the plausible unwanted hydrostannylation of the yne function (*44*) was also fully prevented. After purification by silica gel chromatography, the deoxygenated **6** whose structure was confirmed by ^1H and ^{13}C NMR spectra was obtained in a 56% (pure) yield.

Removal of the silyl protecting group from **6** was then easily achieved by treatment with ~5 mol equiv of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran for 30 min at ambient temperature. This afforded the desired deprotected ferrocene–2′,3′-dideoxyuridine Fc-ddU in excellent yield. Full characterization of the compound is described in the Experimental Section. All physical data confirm the structure.

Scheme 4[a]



[a] (a) β-Cyanoethyl phosphate, DCC, pyridine; (b) aq NH_3 (c) DCC, morpholine, *t*-BuOH/H_2O; (d) $(HNBu_3)_2H_2P_2O_7$, DMSO.

5′-Phosphorylated Ferrocene-2′,3′-dideoxyuridines. Several methods have been described for the chemical preparation of nucleoside triphosphates in the literature (*31*). Most of them involve the coupling of a pyrophosphate with an activated nucleoside monophosphate. Of the commonly used methods a priori compatible with the ferrocene subunit, we judged that the Moffatt phosphomorpholidate coupling approach (*45*) would offer the mildest and most practical route to ferrocene–nucleoside triphosphate Fc-ddUTP **11** from **7** via the monophosphate derivative **9** (Scheme 4).

All phosphorylation reactions (**7** to **11**) were monitored by RP-HPLC, and chemical modifications were characterized by ^{31}P NMR spectroscopy of the products in crude and/or isolated pure forms. The free 5′-hydroxyl group of Fc-ddU was first phosphorylated by condensation with β-cyanoethyl phosphate (*46*) in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) in anhydrous pyridine to afford the 5′-(β-cyanoethyl phosphate) sodium salt intermediate **8** in essentially quantitative yield. The ^{31}P NMR spectrum of **8** obtained after purification on DEAE cellulose anion-exchange chromatography showed one signal at 0.96 ppm corresponding to α-phosphorus. The β-cyanoethyl group of the intermediate **8** was removed in concentrated ammonia at 55 °C for 1 h to afford crude ferrocene-nucleoside 5′-phosphate **9**. The presence of two doublets centered at −8.7 ppm and −13.2 ppm in the ^{31}P NMR spectrum of the reaction mixture suggested the formation of the ferrocene–nucleoside diphosphate as a minor byproduct (<5%). Purification of the product on DEAE cellulose followed by chromatography on DOWEX 50 WX4 cation-exchange resin yielded the morpholinium salt of **9** (58% yield, based on starting **7**, UV–visible determination) which was used without further purification. The presence of the 5′-monophosphate group was proved by the singlet found at 2.1 ppm in the ^{31}P NMR spectrum of **9**. The structure of compound **9** was verified by ^1H NMR spectroscopy and confirmed by its negative-ion FAB spectrum which displayed the $[M - H]^-$ ion at *m/z* 504 as the base peak.

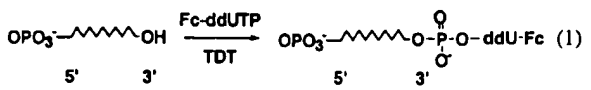
Monophosphate **9** was then converted into its activated 5′-phosphomorpholidate derivative **10** in quantitative yield by refluxing with morpholine in the presence of DCC in aqueous *tert*-butyl alcohol (*45*). In agreement with the proposed intermediate **10** (Scheme 4) was the observation of a new singlet at 4.9 ppm in the ^{31}P NMR spectrum of the crude product obtained after an aqueous workup procedure (*45, 47*). The solid was rigorously dried and treated with a 5 equiv of commercial tri-*n*-butylammonium pyrophosphate ($(HNBu_3)_2H_2P_2O_7$) in dry DMSO for 3 days. This yielded a crude product that was first purified by anion-exchange chromatography to yield a crude sample of ferrocene nucleoside 5′-triphosphate, Fc-ddUTP, **11**. Compound **11** could be further purified and desalted by HPLC using the volatile triethylammonium acetate (TEAA) buffer as eluant.

Chromatography thus provided spectroscopically pure **11** as a triethylammonium salt in 48% yield. In particular, the ^{31}P NMR spectrum only showed the three characteristic phosphorus atom signals due to the triphosphate chain with the expected P–P geminal coupling. The structure of compound **11** was also confirmed by its negative-ion FAB-MS.

Synthesis of 5′-Phosphorylated Oligonucleotides Containing a 3′-Terminal Ferrocene Label. To obtain uniformly successful results in 3′-end-tailing experiments, an important practical problem we studied preliminarily was the quality of the starting single-stranded 5′-phosphate-labeled oligonucleotide products supplied by ODN manufacturers. As it is known, the purity of crude unlabeled synthetic oligonucleotides depends on the length of the sequence to synthesize. The main impurities that are difficult to separate from (*n*)-mer (*n* nucleotides in length) are the $n - 1$ and $n - 2$ congener species (called runts of synthesis). In addition to these contaminants, chemical phosphorylation, which is achieved during solid-phase oligonucleotide syntheses using phosphoramidites, is often nonquantitative. Runts and unphosphorylated 5′-OH-ODN sequences cannot be eliminated through a simple desalting step and should be removed using purification methods. After reversed phase HPLC or ion-exchange HPLC, the 5′-modified oligonucleotide with the correct length and label is usually provided from the manufacturer as a HPLC-grade product.

Therefore, we developed a fast and informative C_{18} RP-HPLC analytical method for routine quality control of these 5′-modified synthetic products (up to 20 bases). HPLC traces A and B in Figure 2 clearly illustrate the high degree of variability in quality encountered among two HPLC-grade 5′-phosphorylated ODNs differing only in their commercial origin. As evidenced from HPLC profiles, some products regarded by their manufacturers as "gold standard" are actually unsuitable samples for most experiments due to high contamination with the parent 5′-hydroxylated sequence (slower-eluting species, trace A) and not well-resolved secondary structures. Nevertheless we have found that HPLC-grade 5′-phosphorylated products from ESGS Cybergene were practically free of impurities, their purity being >85% (Figure 3, trace B) in complete agreement with the analytical certificate of the provider.

An optimized 3′-ferrocene end-labeling procedure using terminal deoxynucleotidyl transferase TDT was set up at the 20 nmol scale of a HPLC-purified 5′-phosphorylated oligonucleotide of defined length (10-mer 5′-p-$(dT)_{10}$ sequence, ~2 OD_{260} units) by adaptation of the standard protocol reported by Roychoudhury and Wu (*48*). The enzymatic incorporation reaction of the redox-labeled dideoxynucleotide Fc-ddUTP at the 3′-terminus of the oligonucleotide (eq 1) was analyzed by C_{18} RP-HPLC



using a linear gradient of acetonitrile in phosphate buffer,

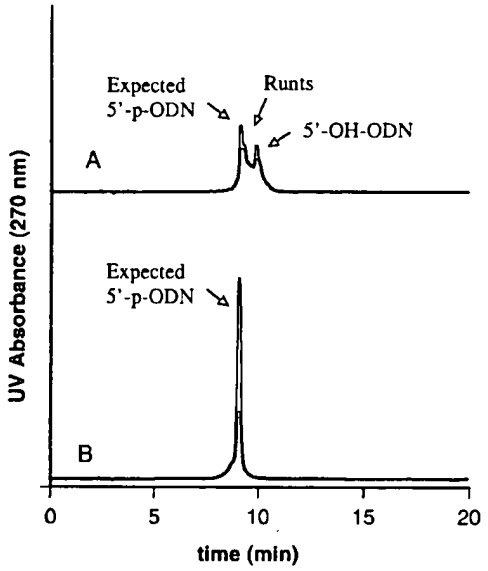


Figure 2. RP-HPLC Profiles of Commercial HPLC-Purified Grade 5′-Phosphorylated Oligonucleotides. 5′-p-$(dT)_{20}$ sample provided (A) from Genset (B) from ESGS Cybergene. Column: Nucleosil C_{18}, 120 Å; eluant A: 10% acetonitrile in 0.1 M aqueous triethylammonium acetate, pH 7.0; solvent B, acetonitrile, linear gradient 2.5–25% B in 30 min, flow-rate 0.8 mL /min.

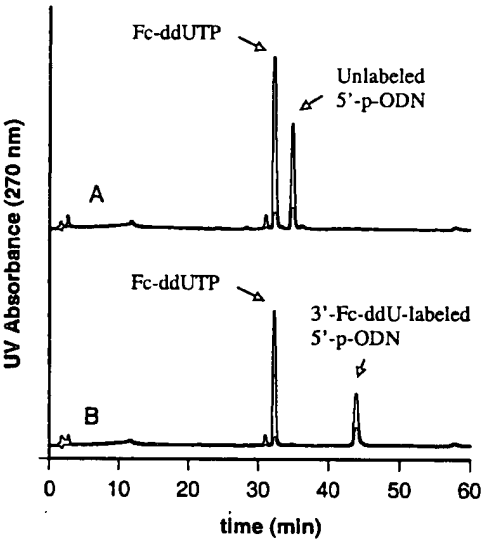


Figure 3. Analytical HPLC Control of 3′-End-labeling of 5′-Phosphorylated Oligonucleotide with Fc-ddUTP. Reaction mixture 200 nmol Fc-ddUTP + 20 nmol 5′-p-$(dT)_{10}$ in enzymatic buffer at 37 °C: (A) before TDT addition; (B) 30 min after TDT addition and after first Centricon-30 membrane ultrafiltration. Column: Novapak C_{18}, 60 Å; solvent A, 10% acetonitrile in aqueous KH_2PO_4 (20 mM) and $NBu_4KH_2PO_4$ (5 mM), pH 7.5; solvent B, acetonitrile, linear gradient 10–40% B in 60 min, flow-rate 0.8 mL/ min.

at different times, after removal of the TDT enzyme by ultrafiltration on 30000 molecular weight cutoff membrane. As demonstrated in Figure 3, a rapid and complete ferrocene-labeling of the 10-mer sequence (trace A, retention time 34.4 min) could be achieved when using approximately 10-fold molar excess of Fc-ddUTP (traces A and B, retention time 32.0 min). Under our analytical reversed-phase HPLC conditions, the oligonucleotide extended at its 3′-end by a single hydrophobic ferrocene-nucleoside label Fc-ddU (trace B, retention time 43.9 min) was a quite slower-eluting species than Fc-ddUTP. This interesting chromatographic separation feature was also obtained at the preparative scale level using a similar appropriate chromatographic system (see Experimental Section for details), and thus made purification of the ferrocenylated-oligonucleotide from excess Fc-ddUTP and other fastest eluting reaction contaminants a facile and efficient step. The final yield in ferrocenylated-oligonucleotide 5′-p-$(dT)_{10}$-ddU-Fc-3′ product was about 75–80% (based on starting oligonucleotide) as determined from HPLC peak integral, and purity was confirmed by HPLC quality control method. Finally, the ferrocenylated-oligonucleotide sample was desalted from the chromatographic phosphate salts by RP-HPLC with volatile TEAA buffer and lyophilized. The identity of the Fc-ddU-terminated oligonucleotide product was confirmed by matrix-assisted laser desorption–ionization/time-of-flight (MALDI-TOF) mass spectrometry in the negative mode, using the carboxylic acid 3-hydroxypicolinic acid (3-HPA)/ diammonium citrate as a useful two component matrix for detection of the molecular ion peak, $(M - H)^-$ of labeled 5′-p-$(dT)_{10}$ (*49*).

In conclusion, by starting from the readily available 5-iodouridine and ferrocene–alkyne **2** we developed an efficient synthetic approach for preparing the first ferrocene-labeled dideoxynucleotide Fc-ddUTP **11**. Although the overall yield of Fc-ddUTP cannot be regarded as outstanding (<10%), the methods presented here are reliable and all ferrocene intermediates can be easily purified. It is worth emphazing that the ferrocene–dideoxynucleoside intermediate Fc-ddU **7** can be prepared in gram batches and that the target compound Fc-ddUTP can be obtained pure in practical quantities for incorporation into single-stranded DNA. Our results also clearly show that enzymatic 3′-ferrocene end-labeling of small 5′-phosphorylated synthetic oligonucleotides with TDT using Fc-ddUTP as a substrate is a highly efficient reaction which can be extended with great promise to larger and more complex 5′-modified oligomers. Finally, by proving that significant quantities of 3′-ferrocene-labeled oligonucleotides can be prepared, this work sets the stage for our studies of various redox-labeled DNA probes required for attaching monolayers of interesting redox-active oligonucleotide on surfaces.

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